Subcellular Distribution of Protein Kinase C in Rat Colonic Epithelial Cells with Different Proliferative Activities

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ABSTRACT

Activation of Ca2+ and phospholipid-dependent protein kinase C (PKC) is associated with increased proliferation in several cell types. When activated, PKC is tightly bound to the particulate cell fraction. Accordingly, we examined the subcellular distribution of PKC in superficial (nonproliferative) and proliferative colonic epithelial cells from rat colonic mucosa. PKC was determined in soluble and particulate fractions of these cells following partial purification of enzyme activity of cellular homogenates by DEAE-cellulose chromatography. In the superficial cells, 90% of the PKC was associated with the soluble fraction. By contrast only 42% of the enzyme activity was found in the soluble fraction of proliferative cells. The specific activity of protein kinase C was higher in the particulate fraction of proliferative compared to superficial cells when expressed as a function of either particulate protein or cellular DNA content. Addition of deoxycholate or 12-O-tetradecanoylphorbol-13-acetate induced a translocation of protein kinase C from the soluble to the particulate fraction. [3H]Thymidine incorporation into DNA was higher in colonic epithelial cells isolated from the colons of rats which had been exposed to deoxycholate or 12-O-tetradecanoylphorbol-13-acetate in vivo. Treatment of rats with 1-(5-isoquinolinyl)-2-methylpiperazine (H-7) suppressed basal [3H]thymidine incorporation into DNA and increases in this parameter induced by 12-O-tetradecanoylphorbol-13-acetate and deoxycholate. The results are consistent with a positive role for activation of protein kinase C in the control of colonic epithelial proliferation.

INTRODUCTION

We have recently provided evidence that activation of protein kinase C is involved in the stimulation of colonic epithelial proliferative activity by bile salts and tumor-promoting phorbol esters (1). Thus, activation of protein kinase C in vivo by bile salts, as assessed by enzyme translocation from the soluble to the particulate fraction of colonic epithelium, was correlated with bile salt-induced increases in ornithine decarboxylase activity and [3H]dThd incorporation into DNA. Moreover, TPA and OAG, which stimulate protein kinase C directly, also increased proliferative activity of colonic epithelium in vivo. The ability of DOC and TPA to stimulate colonic proliferative activity was correlated with their ability to induce the translocation of protein kinase C to the particulate fraction over a wide range of concentrations of DOC and TPA (1). We have previously described a procedure, based on that of Quill and Weiser in small intestine (2), for the isolation of rat colonic epithelial cells with different proliferative activities (3) and have characterized cyclic nucleotide metabolism and the profile of cyclooxygenase and lipoxygenase products in these isolated cell pools (3-5). The present report compares the activity and subcellular distribution of protein kinase C in isolated superficial (nonproliferating) and proliferative colonic epithelial cells. Since deoxycholate (DOC), other bile salts, and TPA were previously shown to increase ornithine decarboxylase activity and to enhance [3H]dThd incorporation into colonic mucosal DNA in vivo (1), we also examined the influence of DOC and TPA on the subcellular distribution of protein kinase C activity in isolated colonic epithelial cells. The effects of treatment of rats with H-7, an inhibitor of protein kinase C activity (6) on [3H]dThd incorporation into colonic epithelial cell DNA, was also assessed. The results support the possibility that activation of protein kinase C may serve as a positive intracellular signal to colonic epithelial cell growth.

MATERIALS AND METHODS

Isolation and Incubation of Superficial and Proliferative Colonic Epithelial Cells. Female Sprague-Dawley rats (Zivic-Miller Laboratories, Pittsburgh, PA) were injected i.p. with 300 μCi/kg [3H]dThd. After 1 h rats were anesthetized with pentobarbital (50 mg/kg) and the distal colons were removed and placed in chilled saline containing 5 mg% gentamicin and 30 mg% penicillin. Colonic epithelial cells of different proliferative activities were isolated by a sequence of five timed incubations as described in detail (2, 3) except that fetal bovine serum was omitted. The colons were lavaged, everted, filled with 0.85% NaCl and tied at both ends with surgical suture. Each colonic loop was incubated in a 50-ml flask with 10 ml of Media 199, bicarbonate buffer containing 2 mM EGTA, 30 mg% penicillin, and 5 mg% gentamicin, equilibrated with 95% O2/5% CO2. After a preliminary incubation for 15 min at 37°C that resulted in release of a number of cells which did not exclude trypan blue, the colonic epithelial cells were isolated by timed sequential incubation of the colonic loops in the same buffer with additions and for the times indicated as follows: incubation I, 2 mM dithiothreitol, 40 min; II, 27 mM citrate, 15 min; III, 2 mM dithiothreitol, 15 min; IV, 2 mM dithiothreitol, 30 min, V, 2 mM dithiothreitol, 30 min. Cells obtained in the first two timed incubations were combined and designated superficial cells. Cells obtained in the fourth and fifth incubation were combined and designated proliferative cells. Pooled superficial and proliferative cells were washed three times and resuspended in Krebs-Ringer bicarbonate buffer containing 1 mg/ml glucose and 1.5 mM CaCl2, equilibrated with 95% O2/5% CO2. The superficial and proliferative cell pools were incubated separately for 30 min at 37°C at the same cell density (6 x 10⁶ cells/ml) in a shaking incubator. Where indicated in the "Results," test agents were added during the final 10 min of incubation. Washed cell isolates were stained with hematoxylin and eosin. More than 90-95% of the cells were epithelial cells by light microscopy. The viability of each preparation was assessed by trypan blue exclusion and was routinely greater than 95%. A portion of the cells from each experiment were used for determination of [3H]dThd incorporation into DNA as previously described (3).

Isolation of Protein Kinase C. At the end of 30 min the incubates were centrifuged at 3500 x g, the pellets resuspended, and then homogenized with a motor-driven teflon pestle in 5 ml of 20 mM Tris, pH 7.5, 0.5 mM EGTA, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride plus 0.5 mM benzamidine (buffer A) as previously described in detail (1). The homogenate was centrifuged at 100,000 x g and the supernatant fraction saved for DEAE-cellulose chromatography. The 100,000 x g pellet was resuspended in buffer A plus 0.2% Triton X-100, mixed on ice for 60 min, and centrifuged at 100,000 x g. The 100,000 x g soluble and solubilized particulate fractions were then applied to DEAE-cellulose columns (0.5 x 4.5 cm) which had been
equilibrated in buffer A. The columns were washed with 20 ml of buffer A and eluted with a 40 ml linear 0–0.07 M NaCl gradient in the same buffer. Eluted fractions were assayed for protein kinase C activity in the presence and absence of 80 μg/ml (100 μM) of phosphatidylserine as described below.

Assay of Protein Kinase C Activity. Protein kinase C activity was determined as previously described (1). Reaction mixtures contained 20 mm Tris, 10 mm MgCl₂, 400 μg/ml lysine-rich histone (Sigma type III-S), 50 μM [³²P]ATP (1 μCi), 1 mm CaCl₂, and where indicated 80 μM phosphatidylserine in a final volume of 75 μl. The contributions of EGTA and EDTA to the assay mixture from the column elution buffer were 0.17 and 0.66, respectively. Ca²⁺ was routinely present in the assay mixture at 1 mm in excess of EDTA plus EGTA. Incubations were for 5 min at 30°C. Reactions were stopped by pipeting 50 μl of the assay mixtures onto a square (1 x 1 cm) of filter paper (Whatman 31) that had been dipped in 10% trichloroacetic acid, 2 mm NaH₂PO₄. Filter papers were washed with agitation in 250 ml of ice-cold trichloroacetic acid for 15 min followed by four changes of 10% trichloroacetic acid at room temperature. The papers were soaked in 95% ethanol for 5 min followed by ether for an additional 5 min and allowed to air dry before counting. Protein kinase C activity is defined as the difference between activity measured in the presence and absence of phosphatidylserine. Enzyme activity was linear from 2 to 5 min under all conditions of assay employed.

Intracolonic Instillation of Test Agents. Rats were fasted for the previous 8 h and throughout the study period and anesthetized with pentobarbital, 50 mg/kg i.p. The large intestine was exposed through a mid-line abdominal incision. A 20-gauge needle was inserted into the lumen of the large intestine at the cecal junction to prevent escape of test solutions. Saline (2 ml), or a solution of test agents in 2 ml of saline (37°C) was instilled into the lumen of the distal colon. In some studies as indicated in the text, 30 min after the insertion of the first test solution, a second instillation of 2 ml of saline or a second test agent in saline (37°C) was made. The needle puncture site was then oversewn. Twenty-three h after the second instillation, rats were injected with [³H]dThd 300 μCi/kg i.p. One h after [³H]dThd injection rats were sacrificed and proliferative colonic epithelial cells were isolated as described above.

Statistics. Statistical significance of differences between mean values was determined by the t test for unpaired data. In vitro incubations for the determination of protein kinase C activity were performed in duplicate unless otherwise indicated. Each experiment was performed three times. The average value of duplicate determinations from each experiment was entered as a single value for the purposes of statistical analysis (N = 3; degrees of freedom = 4 comparing any two experimental conditions by independent t test). In vivo studies were conducted on five rats in each experimental group. For the purposes of statistical analysis the average of duplicate determinations from a single rat colon was entered as a single value (N = 5; degrees of freedom = 8 comparing any two conditions by independent t test).

RESULTS

Fig. 1 illustrates the partial purification by DEAE-cellulose chromatography of protein kinase C from soluble and solubilized particulate fractions of homogenates of isolated superficial and proliferative colonic epithelial cells. Consistent with results obtained in other tissues (7, 8), enzyme activity was not detectable prior to DEAE-cellulose purification possibly due to the presence of phospholipid-insensitive kinases in the crude preparations or to the removal of an endogenous inhibitor. Protein kinase C activity eluted in a sharp peak between 0.035 and 0.050 M NaCl. Activity was not detectable in the absence of Ca²⁺ and was stimulated approximately 5-fold by phosphatidylserine.

Table 1 illustrates the subcellular distribution of protein kinase C activity in superficial and proliferative colonic epithelial cells. As shown, the in vivo rate of [³H]dThd incorporation into DNA was approximately 6-fold higher in proliferative compared to superficial cell pools employed for the studies of protein kinase C activity. Total soluble protein kinase C activity of proliferative cells was about 25% of that seen in superficial cells obtained from the same rat colons. By contrast, particulate enzyme activity of proliferative cells was 3-fold higher than that of the superficial cells. The proportion of protein kinase C activity in the soluble fraction was thus 90% in the superficial cells and only 42% in the proliferative cells. The higher levels of protein kinase C activity in the particulate fraction and lower levels in the soluble fraction of proliferative compared to the respective values in the superficial cells were not a reflection of different protein content in the fractions of these cell pools. Thus, the differences in subcellular distribution of the enzyme were also evident when enzyme activities were expressed as a function of soluble or particulate protein (Table 1) or as a function of total cellular DNA.

Table 2 shows the effects of DOC and TPA on the subcellular distribution of protein kinase C activity in superficial and proliferative colonic epithelial cells. In these studies test agents were incubated with the intact isolated superficial or proliferative cells for 10 min at 37°C at the final concentrations shown in the table. As illustrated, DOC (1–5 mm) markedly decreased the percentage of soluble protein kinase C activity in both superficial and proliferative cells to the same low level. The reduction in soluble activity was accounted for by an increase in activity associated with the particulate fraction, implying a translocation of enzyme activity from the soluble to the particulate fraction rather than loss of enzyme activity. As is also shown in Table 2, incubation of isolated cells with the tumor-promoting phorbol ester TPA resulted in a reduction in percentage of soluble protein kinase C activity in both superficial and proliferative cells. By contrast, 4a-phorbol-12,13-didecanoate and phorbol, which do not promote tumor formation or stimulate the proliferation of colonic epithelium in vivo (1), did not alter the subcellular distribution of protein kinase C activity in these isolated cells.

Table 3 shows the effects of intracolonic instillation of H-7 on [³H]dThd incorporation into DNA of proliferative colonic epithelial cells basally and following stimulation with TPA or DOC. In these studies rats were treated intracolonically with saline, TPA, or DOC in the presence and absence of H-7 for the times indicated in the table. Rats were then injected with [³H]dThd (300 μCi/kg i.p.). Colonic epithelial cells were isolated from the proliferative region of the colonic crypt and [³H]dThd incorporation into DNA determined in the isolated cells. The concentrations of TPA (10 μM) and DOC (5 mm) employed in these in vivo experiments were previously shown to increase mucosal ornithine decarboxylase activity, stimulate [³H]dThd incorporation into DNA of mucosal scrapings, and induce translocation of protein kinase C to the particulate fractions of mucosal homogenates when administered by intracolonic instillation (1). As illustrated in Table 3, analogous to results obtained with mucosal scrapings, [³H]dThd incorporation into DNA of epithelial cells isolated from the colons of rats exposed to TPA or DOC was higher than that in cells isolated from colons of rats receiving saline alone. As is also shown in Table 3, intracolonic instillation of H-7 suppressed basal [³H]dThd incorporation into DNA and increases in this parameter induced by TPA and DOC.

DISCUSSION

The colonic epithelium undergoes an orderly process of migration and renewal. Cells from the lower crypt migrate upward
to replace superficial cells which have been sloughed (9, 10). As the cells in the deeper portions of the crypt migrate towards the surface they differentiate into mature cells and lose their capacity to synthesize DNA. In certain preneoplastic states (11-13) and following chronic exposure to bile salts, under conditions which lead to tumor promotion (14), the colonic epithelial cells do not lose their capacity to synthesize DNA as they migrate towards the surface. The factors which control cell migration and differentiation in the normal colon and the mechanisms mediating an abnormal extension of the proliferative zone are not known.

In some cell systems there appears to be a reciprocal relationship between cAMP-dependent protein kinase and Ca2+-phospholipid-dependent protein kinase C during cellular replication (7). Thus, cAMP and cAMP-dependent protein kinase C activity were reduced and protein kinase C was activated during periods of enhanced growth after cell dilution, following viral transformation and during passage of cells through the
PROTEIN KINASE C IN COLONIC EPITHELIAL CELLS

Table 3 Effects of H-7 on [3H]dThd incorporation into DNA of proliferative colonic epithelial cells

<table>
<thead>
<tr>
<th>Instillation 1</th>
<th>Instillation 2</th>
<th>[3H]dThd incorporation (dpm/µg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>Saline</td>
<td>32 ± 4</td>
</tr>
<tr>
<td>Saline</td>
<td>10 mM TPA</td>
<td>51 ± 8*</td>
</tr>
<tr>
<td>Saline</td>
<td>5 mM DOC</td>
<td>62 ± 7*</td>
</tr>
<tr>
<td>5 mm H-7</td>
<td>5 mm H-7</td>
<td>18 ± 3*</td>
</tr>
<tr>
<td>5 mm H-7</td>
<td>H-7 + TPA</td>
<td>24 ± 4*</td>
</tr>
<tr>
<td>5 mm H-7</td>
<td>H-7 + DOC</td>
<td>27 ± 4*</td>
</tr>
</tbody>
</table>

* P < 0.05 compared to corresponding value in rats which did not receive TPA or DOC.

DNA synthetic phase of the cell cycle (7). The results of the current and earlier studies (3) suggest that a reciprocal relationship may also exist between cAMP-dependent protein kinase and protein kinase C activities in superficial and proliferative colonic epithelial cells.

Studies conducted both in vitro and in vivo have suggested roles for prostaglandins and cAMP in the suppression of proliferative activity of colonic mucosa (3, 4, 15, 16). Thus, production of several prostaglandins known to increase cAMP, including PGE2, PGF2α, and PGD2 (4), as well as steady state levels of cAMP (3) are lower in proliferative compared to superficial cells. The state of activation of cAMP-dependent protein kinase activity is also reduced in the proliferative compared to the superficial cells (3). Prostaglandins which increase cAMP, as well as dibuteryl cAMP suppress [3H]dThd incorporation into colonic mucosa in vitro (15). Moreover, treatment of rats with indomethacin or aspirin which inhibit endogenous prostaglandin production and lower mucosal cAMP content increased [3H]dThd incorporation into colonic mucosal DNA when examined both in vivo and ex vivo (15, 16).

In the present study the proportion of protein kinase C activity associated with the particulate fraction was clearly higher in the proliferative cell pools than in nonproliferating superficial cells. This finding implies that a greater proportion of the enzyme activity in the proliferative cells was in an activated state (17). Thus, protein kinase C forms a complex with Ca2+ and phospholipid upon activation in cell free systems (17) and translocates from the soluble to the particulate fraction upon activation in situ in response to tumor-promoting phorbol esters (8, 18), or when exposed to hormonal agents such as thyroid tropo-relaxing hormone (19) which stimulate polyphosphoinositide turnover. In the present study, the factor(s) responsible for the greater degree of activation of protein kinase C in the proliferative colonic epithelial cells were not identified. However, it is clear from our results that protein kinase C from both superficial and proliferative cells could be activated to a similar extent. Thus, addition of DOC or TPA to the isolated cells resulted in a marked shift in protein kinase C activity from the soluble to the particulate fraction. As previously reported, it is likely that DOC activates protein kinase C in colonic epithelial cells indirectly by stimulating polyphosphoinositide turnover (1). By contrast, TPA likely stimulates protein kinase C activity directly by binding to the soluble enzyme and reducing its requirement for Ca2+ and phospholipid (1).

The finding of higher particulate protein kinase C activity in proliferative colonic epithelial cells compared to nonproliferating superficial cells is consistent with a role for protein kinase C in the positive control of cell growth. This notion was further supported in the present study by the finding that treatment of rats with H-7, an inhibitor of protein kinase C (6) inhibited basal proliferative activity in the isolated epithelial cells and suppressed the enhanced proliferative activity observed in epithelial cells isolated from the colons of rats exposed to TPA or DOC. However, these results must be interpreted with caution. H-7 inhibits cAMP- and cyclic GMP-dependent protein kinase activities as well as protein kinase C (6). Although we cannot completely exclude a role for suppression of cyclic nucleotide-dependent protein kinase activation in the mediation of H-7 action on proliferative activity, previous studies of colonic epithelium have indicated that increased cAMP and activation of cAMP-dependent protein kinase is associated with suppression of proliferative activity (3, 15). By contrast, cyclic GMP has not been found to influence colonic proliferative activity (15). Accordingly, it is unlikely that the ability of H-7 to suppress colonic proliferative activity is due to inhibition of cyclic nucleotide-dependent protein kinases.

Epidemiological studies in humans (20, 21) and studies in animals treated with chemical carcinogens (22) have linked the promoting effects of a high fat diet on colon tumorigenesis to increased fecal excretion of bile salts. Studies from our own (23) and other (24) laboratories have demonstrated that a single intracolon instillation of bile salts (1–25 mM, 8–200 µmol/kg) increases the proliferative activity of colonic mucosa. The doses of bile salts which increase proliferative activity encompass (5 mM, 10 µmol/kg) previously shown to promote colon tumor formation in rats in response to a chemical carcinogen when administered five times a week for 13 months (25). These studies raise the possibility that the bile salt-induced increases in proliferative activity render the colonic epithelium more susceptible to colon tumor growth. The concentration of bile salts employed in the present and previous studies (23–26) are not normally found in the colonic lumen but may be encountered in patients who have undergone small bowel resection (27). Moreover, the effects of bile salts to increase proliferative activity are closely correlated with superficial cell sloughing over a wide range of concentrations (26). These observations raise questions concerning the physiological relevance of bile salt actions on colonic proliferative activity. Nevertheless, under certain conditions the ability of bile salts to increase proliferative activity can be prevented without altering bile salt-induced superficial cell loss (26). The latter studies have suggested that factors in addition to superficial cell loss are involved in the expression of the proliferative response. In addition, we have identified three agents TPA, OAG, and arachidonic acid which increase colonic proliferative activity and cause translocation of protein kinase C to the particulate fraction without causing superficial cell loss or damage (1, 28).

Of note, earlier studies in bile fistula rats have demonstrated a reduction in colonic proliferative activity following diversion of bile from the colon lumen (29), supporting a physiologic role for bile in the maintenance of basal mucosal proliferative activity. It is possible that local concentrations of bile salts may be higher than that determined from studies of bulk fecal extraction (27). Clearly additional studies will be required to determine the physiological significance of bile salt action on proliferative activity.

A number of growth factors including epidermal growth factor, platelet-derived growth factor, and insulin have been reported to enhance polyphosphoinositide turnover in target cell systems (30–34). It is possible that one or more of these growth factors could play a role in the activation of protein

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protein kinase C in proliferative colonic epithelial cells. The influence of these factors on polyphosphoinositide turnover or protein kinase C activity have not been examined in superficial and proliferative colonic epithelium but these studies would clearly be of interest. Lipoxygenase products including LTB₄ (35), lipoxins (36), and certain hydroxy fatty acids (36) have also been reported to stimulate polyphosphoinositide turnover or activate protein kinase C in other cell systems. In this regard, it is of interest that we have previously found enhanced formation of lipoxygenase products in isolated proliferative relative to superficial colonic epithelial cells (5). It is thus possible that enhanced generation of lipoxygenase products in proliferative cells may be responsible for the greater degree of activation of protein kinase C in these cells.

ACKNOWLEDGMENTS

The authors are indebted to Karen Thornburg for excellent technical assistance and to Kathy Daum for secretarial support.

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